JOURNAL OF BACTERIOLOGY, Oct. 1995, p. 5918–5923 Vol. 177, No. 20
0021-9193/95/$04.00+0

Kinetic Characterization of 4-Amino 4-Deoxychorismate Synthase from *Escherichia coli*

V. K. VISWANATHAN, JACALYN M. GREEN,† AND BRIAN P. NICHOLS*

Laboratory for Molecular Biology, Department of Biological Sciences, The University of Illinois at Chicago, Chicago, Illinois 60607-7060

Received 1 May 1995/Accepted 28 July 1995

The metabolic fate of *p*-aminobenzoic acid (PABA) in *Escherichia coli* is its incorporation into the vitamin folic acid. PABA is derived from the aromatic branch point precursor chorismate in two steps. Aminodeoxychorismate (ADC) synthase converts chorismate and glutamine to ADC and glutamate and is composed of two subunits, PabA and PabB. ADC lyase removes pyruvate from ADC, aromatizes the ring, and generates PABA. While there is much interest in the mechanism of chorismate aminations, there has been little work done on the ADC synthase reaction. We report that PabA requires a preincubation with dithiothreitol for maximal activity as measured by its ability to support the glutamine-dependent amination of chorismate by PabB. PabB undergoes inactivation upon incubation at 37°C, which is prevented by the presence of chorismate or PabA; glutamine enhances the protective effect of PabA. Incubation with fresh dithiothreitol reverses the inactivation of PabB. We conclude that both PabA and PabB have cysteine residues which are essential for catalytic function and/or for subunit interaction. Using conditions established for maximal activity of the proteins, we measured the K_m values for the glutamine-dependent and ammonia-dependent aminations of chorismate, catalyzed by PabB alone and by the ADC synthase complex. Kinetic studies with substrates and the inhibitor 6-diazo-5-oxo-L-norleucine were consistent with an ordered bi-bi mechanism in which chorismate binds first. No inhibition of ADC synthase activity was observed when *p*-aminobenzoate, sulfanilamide, sulfathiazole, and several compounds requiring folate for their biosynthesis were used.

Chorismate is a branch point precursor in the biosynthesis of several aromatic compounds in *Escherichia coli*. The compounds that derive from chorismate are *p*-aminobenzoate (PABA), tryptophan, tyrosine, phenylalanine, menaquinone, ubiquinone, and enterochelin (reviewed in reference 18). The pathways leading to the biosynthesis of some of these compounds, especially the aromatic amino acids, are relatively well characterized, as are the regulatory mechanisms that operate in these systems (reviewed in reference 27). In contrast, little is known about the kinetics of PABA biosynthesis.

PABA, an intermediate in the tetrahydrofolate biosynthetic pathway in *E. coli*, is synthesized from chorismate in two steps (10, 17). The first step, catalyzed by 4-amino 4-deoxychorismate (ADC) synthase, converts chorismate and glutamine to ADC and glutamate (1, 8–10, 25, 29). ADC synthase is composed of dissimilar subunits, PabA and PabB. In the absence of PabA and glutamine, PabB can convert chorismate to ADC if an excess of ammonia is provided. Elimination of the pyruvyl moiety, with the concomitant aromatization of the ring of ADC, is catalyzed by the pyridoxal phosphate-containing enzyme ADC lyase (8, 9, 20).

Various lines of evidence indicate that physical interaction between the two subunits of ADC synthase is a prerequisite for productive catalysis. The two subunits coelute from a gel filtration column under certain conditions (20). PabA can convert glutamine to glutamate (glutaminase activity) only in the presence of stoichiometric amounts of PabB (22). The kinetic data described in this paper provide additional evidence for subunit interaction.

Chemical modification experiments implicate several residues in PabA and PabB to be important for catalytic activity (15). Site-directed mutagenesis of PabA provides more direct evidence for the mechanistic relevance of the conserved residues Cys-79, His-168, and Glu-170 (23). While some kinetic studies have been performed on the PabB-catalyzed ammonia-dependent amination of chorismate, there has been little research on the glutamine-dependent reaction. In this report, we show that cysteine residues are important for catalytic activity of both PabA and PabB. We have also measured the K_m values for the substrates for the glutamine- and ammonia-dependent amination of chorismate by PabB in the presence and absence of PabA.

The reactions of PABA biosynthesis share some striking similarities with anthranilate (*o*-aminobenzoate) biosynthesis (Fig. 1). Evidence from sequence analysis clearly indicates that the two enzymes are evolutionarily related. While the *E. coli* PabA and TrpG cannot substitute for one another, in some bacteria, a common glutamine amidotransferase subunit participates in both anthranilate and *p*-aminobenzoate biosyntheses (5, 13, 24). The experiments discussed in this paper allow us to extend the comparison between these two enzymes.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* BN117 [*pabA1 pabB*::Kn *pheA1 tyrA4 trpR* (Tn*10*) Δ*trpEA2 his4 proA2 argE3 rpsL704*] transformed with either the plasmid pSZD51 or pSZD52 was used to overproduce PabA (~550-fold) and PabB (~250-fold), respectively (17). Transformations were performed as described previously (2). *E. coli* BN117 transformed with pJMG30, a multicopy plasmid expressing *pabC*, was the source of ADC lyase (8). BN117/pSZD52 was grown to the stationary phase prior to harvesting since this enhanced the yield of PabB about threefold. BN117/pSZD51 and BN117/pJMG30 were grown to an optical density of 0.2 prior to harvesting.

Preparation of enzymes. PabB was prepared from extracts of *E. coli* BN117/

* Corresponding author. Mailing address: Laboratory for Molecular Biology, Department of Biological Sciences, 845 West Taylor St., M/c 067, The University of Illinois at Chicago, Chicago, IL 60607-7060. Phone: (312) 996-5064. Fax: (312) 413-2691.

† Present address: Department of Biomedical Sciences, Scholl College of Podiatric Medicine, Chicago, IL 60610.

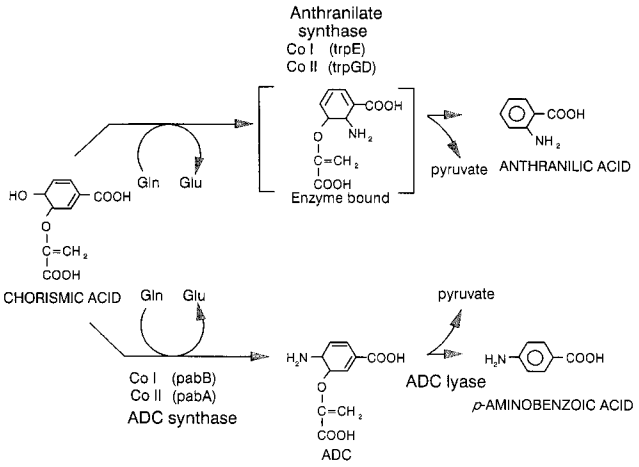

FIG. 1. Reactions of anthranilate and PABA synthesis.

pSZD52. Cells were resuspended in 100 mM Tris-HCl (pH 7.5)–30 mM 2-mercaptoethanol (2 ml/g of cells), disrupted by sonication, and centrifuged at 100,000 $\times$ *g* for 1 h. Streptomycin sulfate was added to the supernatant to a final concentration of 3%. Following stirring for 20 min, insoluble material was removed by centrifugation at 65,000 $\times$ *g* for 15 min. The supernatant was subjected to ammonium sulfate fractionation, and material that precipitated between 30 and 45% saturation was retained. The resulting pellet was dissolved in R buffer (50 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 1 mM EDTA, 10 mM 2-mercaptoethanol, 5% glycerol) and dialyzed extensively against the same buffer. The protein solution was then applied to a DEAE-cellulose column (26 by 1.4 cm) equilibrated with R buffer. The column was washed with 200 ml of R buffer, and the enzyme was eluted from the column with a 0.0 to 0.5 M linear NaCl gradient (600 ml) in the same buffer. Fractions containing maximum activity were combined and dialyzed extensively against R buffer. The specific activity of this material ranged from 0.5×10^3 to 2.0×10^3 U/mg of protein. One unit of enzyme activity is defined as the amount of enzyme required to convert 1 nmol of chorismate to PABA at 37°C in 30 min in the presence of excess PabA and ADC lyase.

For the preparation of PabA, a similarly prepared 100,000 $\times$ *g* supernatant of *E. coli* BN117/pSZD51 was applied to a Sephacryl-S200 column (2.5 by 111 cm) equilibrated with R buffer. Samples containing maximum activity were combined. The activity of this sample ranged from 0.6×10^3 to 1.2×10^3 U/mg of protein.

ADC lyase was partially purified through the yellow-3-agarose step by the protocol described previously (8, 9). Fractions from the yellow-3-agarose column containing maximum enzyme activity were combined and dialyzed against R buffer. The activity of the combined fraction ranged from 1.0×10^6 to 4.0×10^6 U/mg.

Enzyme assays. PabA, PabB, and ADC lyase were routinely assayed as described by Nichols et al. (17). The glutamine-dependent reactions were carried out in 50 mM Tris-HCl (pH 7.5)–5% glycerol–5 mM dithiothreitol (DTT)–5 mM $MgCl_2$–100 μM chorismate–5 mM glutamine. The ammonia-dependent reaction was assayed in 50 mM triethanolamine (pH 8.5)–5% glycerol–5 mM DTT–5 mM $MgCl_2$–100 μM chorismate–100 mM ammonium sulfate. Assays (0.5 ml) were quenched with 0.1 ml of 1 M HCl and extracted with ethyl acetate, and the amount of PABA formed was determined fluorimetrically as described before (12).

It has long been known that PABA added as a supplement to minimal media containing sulfonamides enables *E. coli* to grow at higher levels of the drug (28). Guay found that overexpression of PabB in *E. coli* provided a high level of resistance to sulfanilamide (11). If sulfonamide resistance is due to elevating cellular levels of PABA, then this implies that PabB is the limiting protein in PABA biosynthesis. We therefore decided to limit PabB in our kinetic experiments.

To study the kinetics of the glutamine-dependent reaction, reaction mixtures (3 ml) contained 50 mM Tris-HCl (pH 7.5), 5% glycerol, 5 mM DTT, 5 mM $MgCl_2$, limiting PabB (3 to 7 U), excess PabA (120 U), ADC lyase (3,000 U), and chorismate (5 to 100 μM) or glutamine (0.77 to 10 mM). Each reaction mixture was preincubated for 90 min at 37°C and initiated with the remaining substrate. Aliquots (0.5 ml) were taken at 5, 10, 15, 20, and 25 min, quenched in 0.1 ml of 1 M HCl, and extracted into ethyl acetate. PABA was determined as already described (12). The initial velocity was calculated from the slope of the line made by the time points.

Inhibition studies using 6-diazo-5-oxo-L-norleucine (DON; Sigma) were performed similarly. DON was added either before or after a 90-min preincubation period. When DON was used as an inhibitor with regard to glutamine, the chorismate concentration was 100 μM, and when DON was used as an inhibitor with regard to chorismate, the glutamine concentration was 2.5 mM.

To study the kinetics of the ammonia-dependent reaction, reaction mixtures (3 ml) contained 50 mM triethanolamine (pH 8.5), 5% glycerol, 5 mM DTT, 5 mM $MgCl_2$, ammonia (NH_4^+ plus NH_3; 62.5 to 250 mM), chorismate (28.6 to 200 μM), PabB (15 to 16 U), and ADC lyase (3,000 U). When present, 400 U of PabA was included. The ammonia-dependent amination of chorismate exhibited linear kinetics in the absence of preincubation (see Fig. 4); therefore, the preincubation step was omitted in these studies. In assays using various concentrations of $(NH_4)_2SO_4$, constant ionic strength of the mixture was maintained by the addition of Na_2SO_4. The final ionic strength of the mixture was not inhibitory to PABA formation. Reactions were initiated by the addition of ammonia (NH_4^+ plus NH_3; 62.5 to 250 mM) as $(NH_4)_2SO_4$ in a volume of 200 μl, and initial velocity was determined from the slope of time points as it was for the glutamine-dependent reaction.

Kinetic data analyses were carried out with the program MacEnzkin-Version 1.02 developed by J. D. Ozeran, S. Burstein, T. Chauncey, H. Taylor, and J. Westley (The University of Chicago, Chicago, Ill.). This program provided the best fit to a hyperbolic function by an iterative least-squares method and also permitted the generation of statistically valid secondary plots of slopes and intercepts against the function of the fixed substrate. Error was propagated in quadrature as described previously (4).

Experiments that required a direct assay for ADC synthase activity were conducted by monitoring the formation of ADC by high-pressure liquid chromatography (HPLC). The assay conditions were identical to those described above, except that ADC lyase was excluded. After incubation at 37°C for 30 min, 50 μl of the assay mix was applied to a Waters Nova-Pak C-18 4-μm column (3.9 by 150 mm). The column was developed with 5% acetic acid, and the elution profile was monitored at 270 nm (9, 29). Chorismate, PABA, and ADC eluted as distinct peaks. The area under the ADC peak was then determined.

RESULTS

Establishment of linear kinetics in the ADC synthase–ADC lyase-coupled reaction system. Control experiments to establish the range of linearity for the coupled ADC synthase-ADC lyase reactions using limiting PabB at various substrate concentrations resulted in the observation that the glutamine-dependent production of PABA was nonlinear and accelerated with time at all concentrations of glutamine and chorismate tested. The rate of PABA formation continued to increase over a 60-min period (Fig. 2), instead of the expected linear accumulation phase; this increase was followed by a decrease in

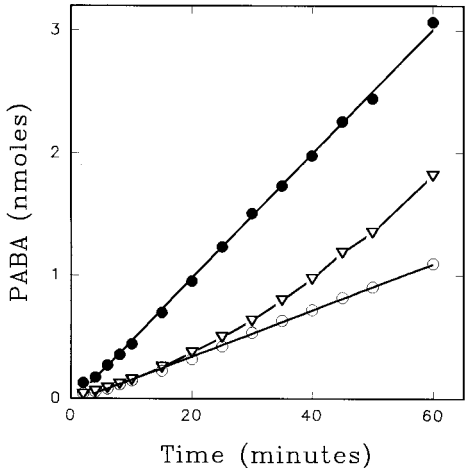

FIG. 2. Effect of preincubation on linearity and rate of PABA formation. All reaction mixtures (10 ml) ultimately contained 50 mM Tris-HCl (pH 7.5), 5% glycerol, 5 mM DTT, 5 mM $MgCl_2$, 100 μM chorismate, 5 mM glutamine, PabB (40 U), PabA (43 U), and ADC lyase (7,700 U). Reaction mixtures were subjected either to no preincubation (open triangles) or to a 90-minute preincubation at 37°C of buffer containing proteins alone (open circles) or containing proteins and 5 mM glutamine (closed circles). Following the preincubation, reactions were initiated by the addition of the remaining assay components, and 0.5-ml aliquots were removed over time and quenched in acid. Product PABA was measured as described in Materials and Methods.

TABLE 1. Reactivation of PabB

Addition to secondary incubation[a]	Initial reaction velocity (pmol/min)
None	13
DTT	52
Chorismate	17
Glutamine	16
PabA	55
PabA and glutamine	41

[a] All samples were subjected to a 90-min primary incubation at 37°C. Aliquots were then subjected to a 30-min secondary incubation at 37°C in the presence of the components indicated. An identical sample which was left on ice for 2 h yielded 42 pmol of PABA per min.

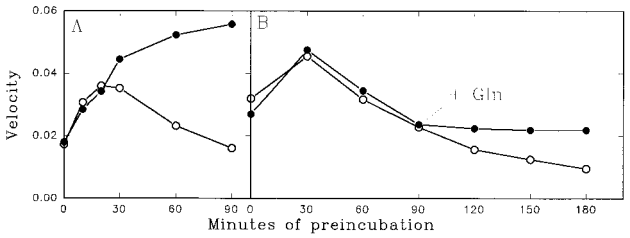

FIG. 3. Effect of glutamine on loss of PabB activity. Reaction mixtures (5 ml) contained 50 mM Tris-HCl, pH 7.5, 5% glycerol, 5 mM $MgCl_2$, 5 mM glutamine, 100 μM chorismate, PabB (7.9 U), PabA (37.5 U), and ADC lyase (3,500 U). (A) Reaction mixtures containing all three proteins with (closed circles) or without (open circles) glutamine were preincubated for the times shown. Reactions were initiated with the remaining substrate(s), and aliquots (0.5 ml) were taken every 2 min, quenched in acid, and analyzed for PABA. The velocity was calculated as the slope of the resulting line (nanomoles of PABA per minute). (B) Parallel reaction mixtures containing all three proteins were preincubated for various times without glutamine in the preincubation (closed circles; open circles up to 90 min). At 90 min, glutamine was added to one set (closed circles), and the preincubation was continued. Reactions were initiated with the remaining substrates, and velocity was calculated as described for panel A.

product formation indicative of substrate depletion. However, preincubation of the reaction mixtures at 37°C for 90 min prior to the addition of substrates resulted in linear accumulation of PABA with time, but at a rate significantly lower than the maximum rate achieved in reaction mixtures that had not been preincubated (Fig. 2). Inclusion of either substrate in the preincubation mixture resulted in linear PABA formation at a significantly higher rate than if the substrate was absent during preincubation (Fig. 2).

A systematic study yielded two basic findings. First, the acceleration phenomenon in which the rate of PABA production increased with time was eliminated by preincubating PabA in the reaction mixture for 90 min. Second, preincubation of PabB at 37°C resulted in an inactivation of PabB, which was responsible for the lower slope observed in Fig. 2.

Purified PabA required an incubation with DTT to achieve maximal activity. When PabA was preincubated for 90 min in the presence of a variety of combinations of reaction components, we found that preincubation of PabA in Tris-HCl (pH 7.5) containing DTT was necessary and sufficient for activation (data not shown). Maximal activation of PabA required DTT at a concentration of 5 mM or higher and a 90-min incubation at 37°C. Glutamine had no effect on the activation of PabA. Most ensuing experiments involving the glutamine-dependent amination of chorismate were performed with PabA which had been preincubated at 37°C for 90 min in buffer containing 5 mM DTT.

PabB was inactivated upon incubation at 37°C. ADC synthase PabB lost activity upon incubation at 37°C (Table 1), but this was prevented when either DTT, chorismate, or PabA was included in the incubation (data not shown). Glutamine alone had no stabilizing effect on PabB. In Fig. 3, which illustrates the experiment in which PabA was not preincubated separately, we can see the combined biphasic effects of PabA activation and PabB inactivation on glutamine-dependent activity in reaction mixtures ultimately containing identical amounts of substrates and enzymes. For the first 30 min, we observed an increase in activity due to activation of PabA by DTT. We observed a loss in PabB activity when glutamine was absent (Fig. 3A); this loss was prevented when glutamine was included in the preincubation. We investigated the effect of an addition of glutamine to a partially inactivated sample (Fig. 3B). We found that while glutamine did not activate inactive PabB, it prevented further loss of activity.

Experiments were then conducted to investigate whether PabB inactivation was reversible. Reversibility would rule out proteolysis and suggest conformational change and/or other local changes as possible modes of inactivation. PabB was incubated in reaction buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 5% glycerol) at 37°C for 90 min. Aliquots of the incubated sample were transferred into several tubes and subjected to a second, 30-min incubation period at 37°C, in the presence of various components of the reaction mixture. The missing components were then added to initiate the reaction, and the initial reaction velocity was determined. PabA was preincubated separately in the presence of fresh DTT prior to reaction initiation. As is evident in Table 1, only DTT was able to reactivate inactivated PabB. Preincubated PabA appeared to reactive PabB, but it is likely that the DTT present in the PabA sample, and not the PabA itself, mediated the reactivation.

PabA stimulated the ammonia-dependent amination of chorismate catalyzed by PabB. All of the experiments discussed above involved the glutamine-dependent amination of chorismate. While PabB alone can catalyze the ammonia-dependent amination of chorismate, the presence of PabA stimulated the reaction (Fig. 4). Interestingly, generation of PABA was linear, even when the reaction mixture was not preincubated and regardless of the presence of PabA.

Determination of the kinetic constants for the ammonia-dependent and glutamine-dependent reactions. Having determined conditions necessary to maintain the full activity of PabA and PabB, we performed experiments to determine the kinetic constants for the glutamine-dependent and ammonia-dependent reactions (Table 2). The K_m value for chorismate varied, depending on whether glutamine or ammonia served as the amino donor and whether PabB or the PabB-PabA complex was catalyzing the reaction measured. The lowest K_m value for chorismate, 4.2 μM, was measured for the PabB-PabA complex as it catalyzed the glutamine-dependent reaction. The K_m value for ammonia (NH_4^+ plus NH_3) likewise varied, increasing about threefold when PabA was present. The double-reciprocal Lineweaver-Burk plots of the initial velocity data for the glutamine-dependent reaction for the PabB-PabA complex showed nonparallel lines that converged to the left and below the axes. Product inhibition studies were not informative because up to 10 mM glutamate exhibited no inhibition, and sufficient amounts of purified ADC were not available. DON, a glutamine analog, inhibited ADC synthase. Inhibition was competitive with respect to glutamine but uncompetitive with respect to chorismate (K_i, ~385 μM). These data were consistent with an ordered bi-bi mechanism for the ADC synthase complex, in which chorismate bound first, followed by glutamine. The order of release of products could not be deduced.

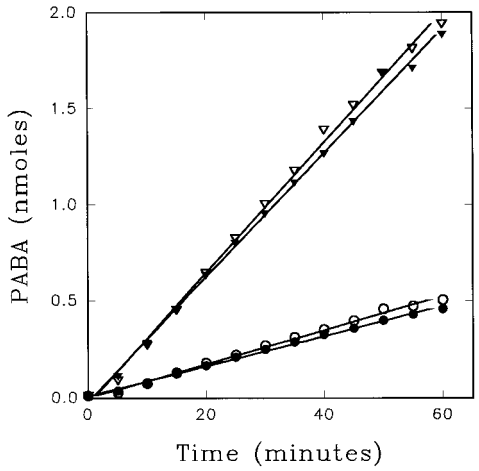

FIG. 4. Effect of PabA and preincubation on the time course of the ammonia-dependent reaction. Reaction mixtures (10 ml) contained 50 mM triethanolamine (pH 8.5), 5% glycerol, 5 mM DTT, 5 mM $MgCl_2$, 64 μM chorismate, 400 mM ammonium sulfate, PabB (94.5 U), ADC lyase (2,300 U), and either 300 U of PabA (open and closed triangles) or no PabA (open and closed circles). Reactions were subjected to either a 90-min preincubation (closed triangles, closed circles) or no preincubation (open triangles, open circles) while containing all components except ammonia. Reactions were then initiated by the addition of ammonia, and 0.5-ml aliquots were taken over time, quenched in acid, extracted, and analyzed for PABA.

There is no evidence of feedback inhibition of ADC synthase by PABA, PABA analogs, or end products of folate metabolism. We studied regulation with the ammonia-dependent reaction under conditions of PabB limitation to ascertain whether various metabolites acted as inhibitors. In separate experiments, different concentrations of ATP, AMP, dAMP, guanosine, GMP, dGMP, GTP, deoxyguanosine, dTMP, serine, glycine, tyrosine, methionine, and *p*-hydroxybenzoate were added to the assay mixture, and the amount of PABA formed was determined. All of the components described above except tyrosine and *p*-hydroxybenzoate require folate derivatives for their biosynthesis. To determine the effect of PABA and the PABA analogs sulfanilamide and sulfathiazole on the ADC synthase reaction, the formation of ADC was monitored in the presence of these compounds by the HPLC method for detecting ADC. None of the compounds had an inhibitory effect on the ADC synthase reaction (data not shown) (26).

DISCUSSION

The enzyme ADC synthase commits chorismate to PABA, and ultimately folate, biosynthesis. It is interesting to analyze the properties of the PABA biosynthetic enzymes in the context of their similarity to the enzyme *o*-aminobenzoate (anthranilate) synthase. While the similarities provide insight into the evolutionary origins of the two enzymes, the differences clearly reflect the unique requirements on the two pathways. Anthranilate synthase consists of two dissimilar subunits, each of which has similarity to the two dissimilar subunits constituting ADC synthase (3, 7, 14). Anthranilate synthase performs a regiospecific amination and subsequent aromatization of chorismate to form the product anthranilate (*o*-aminobenzoate) (30, 31). PABA synthesis requires an additional enzyme, ADC lyase, which does not exhibit any sequence similarity to anthranilate synthase (8). *E. coli* anthranilate synthase is an $\alpha_2\beta_2$ enzyme, consisting of two copies each of TrpE and TrpG. The *trpG* peptide exists as a fusion protein with the *trpD* gene product (reviewed in references 28 and 30). *E. coli* ADC synthase is an αβ dimer and is composed of PabB and PabA (19, 20).

TABLE 2. Summary of kinetic constants for ADC synthase and PabB

Assay[a]	Chorismate K_m (μM)	Gln K_m (mM)	Ammonia[b] K_m (mM)	V_{max} normalized (%)
Gln dependent, PabB-PabA	4.2 ± 1.4	1.60 ± 0.4		100 ± 12
Ammonia dependent				
PabB	83.8 ± 7.5		138 ± 36.0	35 ± 4
PabB-PabA	18.6 ± 7.5		359 ± 86.0	39 ± 9

[a] In the assays, either glutamine or ammonia served as the amino donor, and PabB or PabB-PabA catalyzed the reaction measured. See text for details.
[b] NH_4^+ plus NH_3.

Nearly all work published on the enzymology of PABA biosynthesis has focused on the ammonia-dependent amination of chorismate by PabB, neglecting the contribution of PabA (16, 29). Here we report kinetic data both on the glutamine amidotransferase activity of the PabB-PabA complex and on the ammonia-dependent activity, as catalyzed by the complex and by PabB alone. Control experiments designed to establish conditions of linearity for substrate concentration(s), enzyme concentration(s), and time initially yielded puzzling results. We found that PabA as isolated was partially inactive and required a 90-min incubation at 37°C in the presence of 5 mM DTT to achieve maximal activity, as measured by the ability of PabA to support glutamine-dependent amination of chorismate by PabB. Upon incubation with DTT, PabA showed about a two- to threefold increase in its ability to support the glutamine-dependent amination of chorismate by PabB. Roux and Walsh (22) reported a 10% loss of activity of PabA per day when the enzyme was kept at 4°C in buffer (lacking a reducing agent). In addition, when these researchers incubated radioactive glutamine with the PabB-PabA complex to detect a γ-glutamyl intermediate, they measured only 0.56 mol of acylenzyme per mol of PabB-PabA complex (22). Interestingly, they isolated PabA in the absence of reducing thiols. These results implicate the involvement of cysteine residues in catalysis. Four cysteine residues are conserved across all known PabA sequences. Cys-79, which is analogous to Cys-84 of TrpG, is conserved across all known aminobenzoate amidotransferases. Site-directed mutagenesis of *E. coli pabA*, in which cysteine 79 was replaced by serine, yielded a protein which exhibited low but detectable glutaminase activity (23). Further studies are necessary to determine what role(s) the various cysteine residues might play in ADC synthase activity.

We found that incubation of PabB at 37°C resulted in a progressive loss of activity. This loss was prevented if DTT, PabA, or chorismate was included in the incubation. The protection of PabB by PabA was enhanced by the presence of glutamine; glutamine alone had no effect on PabB activity. This provided kinetic evidence for an association between PabB and PabA. PabB rendered inactive by incubation could be reactivated by a second incubation in the presence of fresh DTT. We conclude that the inactivation of PabB upon incubation is due to oxidation of one or more of the six cysteine residues (7), since the reducing agent DTT can not only prevent but also reverse this loss of activity. Chemical modification studies showed that thiol reagents were inhibitory to PABA biosynthetic activity in cell extracts of *E. coli*, but because the extracts of PabA and PabB each contained contaminating

PabC, it is difficult to conclude which protein(s) was susceptible to the modifying agent (15).

We interpret the requirements of both PabA and PabB for a strong reducing agent to indicate that cysteine residues play important roles in ADC synthase activity. Furthermore, the fact that PabA and chorismate can render PabB stable to oxidation is consistent with cysteine playing an important role at or near the active site of the enzyme.

Having established conditions for performing the enzyme assays, we proceeded to perform a kinetic analysis of ADC synthase and the ammonia-dependent amination of chorismate. The K_m values for glutamine and chorismate are reasonable, both relative to the cellular concentrations of chorismate and glutamine and in comparison with other chorismate interconverting enzymes (Table 2). The K_m values for chorismate of other *E. coli* chorismate-binding proteins are as follows: anthranilate synthase, 1.2 μM; isochorismate synthase, 14 μM; chorismate mutase-prephenate dehydratase, 45 μM; chorismate mutase-prephenate dehydrogenase, 92 μM; and chorismate lyase, 9.7 μM (18). It is interesting to compare the kinetic constants for the glutamine-dependent and ammonia-dependent reactions. The K_m value for chorismate in the ammonia-dependent reaction dropped from 84 to 19 μM when PabA was present. This implies that while PabB alone can bind chorismate productively, the presence of PabA is important in achieving the most efficient conformation for catalysis. In contrast, the K_m value for ammonia (NH_4^+ plus NH_3) actually increases from ~140 to ~360 mM when PabA is present. Perhaps PabA normally acts to channel ammonia from glutamine into the active site and blocks the entrance of exogenous ammonia into the active site. Our kinetic experiments are consistent with an ordered mechanism whereby chorismate binds first, followed by glutamine; this is analogous to the mechanism determined for anthranilate synthase. There is some evidence, however, for a random mechanism, since we have shown that glutamine and PabA can protect PabB from oxidation in the absence of chorismate.

DON binds and inhibits a variety of glutaminases including anthranilate synthase and ADC synthase (22, 30). Roux and Walsh (22) measured a K_i for DON of 210 μM in the glutaminase reaction of ADC synthase. Since the two subunits of ADC synthase (unlike those of anthranilate synthase) do not bind each other avidly, it is customary to use an excess of one of the components and relate the amount of ADC synthase present to the amount of the limiting component. Although DON can alkylate active-site cysteine residues, we did not observe an irreversible loss of ADC synthase activity, possibly because we use an excess of PabA in our experiments. Alkylated PabA on the complex would then equilibrate with the active, unassociated PabA. In our experiments, we found that DON was a competitive inhibitor with respect to glutamine, which is consistent with the two molecules sharing a binding site on ADC synthase.

While it is difficult to separate the two subunits of anthranilate synthase, it has also been difficult to demonstrate interaction between the two subunits of ADC synthase. The two subunits coelute on a gel filtration column under certain conditions (19). PabA has a glutaminase activity in the absence of chorismate but only in the presence of PabB (23). We have demonstrated that while PabB has an aminating activity in the absence of glutamine and PabA, the rate of conversion is stimulated about fourfold in the presence of PabA. The preincubation studies also support the kinetic relevance of subunit interaction. While PabA can protect PabB from inactivation, this effect is enhanced in the presence of glutamine. Glutamine, by itself, does not protect PabB from inactivation. The difference in the K_m values of PabB and the complex (discussed above) provides additional support to the importance of subunit interaction. Preincubation of PabA does not alter its stimulatory effect on the ammonia-dependent amination of chorismate. We interpret this to mean that PabA and PabB can physically interact without this full reactivation and/or reduction occurring. This is consistent with gel filtration experiments performed in our laboratory (19, 20), in which appreciable physical association of PabB and PabA occurred with no preincubation of the subunits.

E. coli PabB and TrpE and *E. coli* PabA and TrpG are 26 and 44% identical, respectively (7, 14). This sequence similarity may reflect a higher order of structural similarity, since polyclonal antibodies raised against anthranilate synthase cross-reacted with fractionated extracts containing PABA synthetic activity (21). While anthranilate synthase is very sensitive to feedback inhibition by tryptophan, ADC synthase was not affected by any of the compounds we studied as potential feedback inhibitors. Mutational studies have identified residues in the N-terminal portion of TrpE as important in binding tryptophan (6), with the catalytic site occupying the carboxyl-terminal portion of the protein (31). Curiously, while the similarity between TrpE and PabB is clearly higher at the carboxyl-terminal portions of the proteins, there remains significant similarity at or near conserved residues in the amino-terminal region that have been shown to be important in feedback inhibition of anthranilate synthase. The conserved N-terminal sequence of TrpE sequences is $X_{(36)}$-LLES-$X_{(10)}$-S. Three feedback-resistant mutations in TrpE are at amino acid residues 39, 40, and 41 (6). PabB proteins from various organisms have the sequence $X_{(32)}$-LL-H/E-S-$X_{(10)}$-D/S at the N terminus. Every PabB protein which contains an H at position 35 has a D at position 47, and each with an E has a corresponding S, implying that these may be conserved hydrogen-bonding couples. Perhaps ADC synthase is regulated by an as-yet-unidentified effector.

ACKNOWLEDGMENTS

This work was supported in part by National Institutes of Health grant GM44199. V. K. Viswanathan was supported by a graduate fellowship from the Laboratory of Molecular Biology, The University of Illinois at Chicago.

We are grateful to John Westley for providing us with MacEnzKin, a software program for analyzing double-reciprocal plots, and also for helpful discussions. We would like to thank Natasha Austria and Dietrich Haas for their technical assistance and Gayatri Vedantam for useful discussions.

REFERENCES

1. **Anderson, K. S., W. M. Kati, Q.-Y. Ye, J. Liu, C. T. Walsh, A. J. Benesi, and K. A. Johnson.** 1991. Isolation and structure elucidation of the 4-amino-4-deoxychorismate intermediate in the PABA enzymatic pathway. J. Am. Chem. Soc. **113:**3198–3200.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. A. Smith, J. G. Seidman, and K. Struhl (ed.).** 1987. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
3. **Baurle, R., J. Hess, and S. French.** 1987. Anthranilate synthase-anthranilate phosphoribosyltransferase complex and subunits of *Salmonella typhimurium*. Methods Enzymol. **142:**366–387.
4. **Bevington, P. R.** 1969. Error reduction and data analysis for the physical sciences. McGraw-Hill Book Co., New York.
5. **Buvinger, W. E., L. C. Stone, and H. E. Heath.** 1981. Biochemical genetics of tryptophan synthesis in *Pseudomonas acidovorans*. J. Bacteriol. **147:**62–68.
6. **Caligiuri, M. G., and R. Bauerle.** 1991. Identification of amino acid residues involved in feedback regulation of the anthranilate synthase complex from *Salmonella typhimurium*. Evidence for an amino-terminal regulatory site. J. Biol. Chem. **266:**8328–8335.
7. **Goncharoff, P., and B. P. Nichols.** 1984. Nucleotide sequence of *Escherichia coli pabB* indicates a common evolutionary origin of *p*-aminobenzoate synthetase and anthranilate synthetase. J. Bacterol. **159:**57–62.

8. **Green, J. M., W. K. Merkel, and B. P. Nichols.** 1992. Characterization and sequence of *E. coli pabC*, the gene encoding aminodeoxychorismate lyase, a pyridoxal phosphate-containing enzyme. J. Bacteriol. **174:**5317–5323.
9. **Green, J. M., and B. P. Nichols.** 1991. *p*-Aminobenzoate biosynthesis in *Escherichia coli*: purification of aminodeoxychorismate lyase and cloning of *pabC*. **266:**12971–12975.
10. **Green, J. M., B. P. Nichols, and R. G. Matthews.** Folate biosynthesis, reduction, and polyglutamylation. In R. C. Neidhardt (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, 2nd ed. American Society for Microbiology, Washington, D.C., in press.
11. **Guay, G.** 1990. Isolation and characterization of novel sulfonamide resistance determinants in *E. coli*. Ph.D. thesis. University of Illinois at Chicago, Chicago.
12. **Huang, M., and F. Gibson.** 1970. Biosynthesis of 4-aminobenzoate in *Escherichia coli*. J. Bacteriol. **103:**767–773.
13. **Kane, J. F., W. M. Holmes, and R. A. Jensen.** 1972. Metabolic interlock: the dual function of a folate pathway gene as an extra-operonic gene of tryptophan biosynthesis. J. Biol. Chem. **247:**1587–1596.
14. **Kaplan, J. B., and B. P. Nichols.** 1983. Nucleotide sequence of *E. coli pabA* and its evolutionary relationship to *trpG*(*D*). J. Mol. Biol. **168:**451–468.
15. **McLeish, M. J., and J. M. Caine.** 1991. Chemical modification of PABA synthase. Biochem. Int. **24:**1033–1042.
16. **McLeish, M. J., P. J. Wookey, and K. G. Mortimer.** 1988. The cloning and overexpression of PABA synthase in *E. coli*. Biochem. Int. **166:**727–735.
17. **Nichols, B. P., A. M. Seibold, and S. Z. Doktor.** 1989. Para-aminobenzoate synthesis from chorismate occurs in two steps. J. Biol. Chem. **264:**8597–8601.
18. **Pittard, A. J.** 1987. Biosynthesis of the aromatic amino acids, p. 368–394. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 1. American Society for Microbiology, Washington, D.C.
19. **Rayl, E. A.** 1993. *Escherichia coli* aminodeoxychorismate synthase: residues involved in catalysis and subunit association. Ph.D. thesis. University of Illinois at Chicago, Chicago.
20. **Rayl, E. A., J. M. Green, and B. P. Nichols.** Unpublished data.
21. **Reiners, J. J., L. J. Messenger, and H. Zalkin.** 1978. Immunological cross-reactivity of Escherichia coli anthranilate synthase, glutamate synthase, and other proteins. J. Biol. Chem. **246:**1587–1596.
22. **Roux, B., and C. T. Walsh.** 1992. *p*-Aminobenzoate synthesis in *Escherichia coli*: kinetic and mechanistic characterization of the amidotransferase PabA. **31:**6904–6910.
23. **Roux, B., and C. T. Walsh.** 1993. *p*-Aminobenzoate synthesis in *Escherichia coli*: mutational analysis of three conserved amino acid residues of the amidotransferase PabA. Biochemistry **32:**3763–3768.
24. **Sawula, R. V., and I. P. Crawford.** 1972. Mapping of tryptophan genes of *Acinetobacter calcoaceticus* by transformation. J. Bacteriol. **112:**797–805.
25. **Teng, C.-Y., B. Ganem, S. Z. Doktor, B. P. Nichols, R. K. Bhatnager, and L. C. Vining.** 1985. Total synthesis of (+/−)-4-amino-4-deoxychorismic acid: a key intermediate in the biosynthesis of *p*-aminobenzoic acid and 1-(*p*-aminophenyl)alanine. J. Am. Chem. Soc. **107:**5008–5009.
26. **Viswanathan, V. K.** 1993. Kinetic characterization of *p*-aminobenzoate biosynthetic enzymes. Ph.D. thesis. The University of Illinois at Chicago, Chicago.
27. **Walsh, C. T., J. Liu, F. Rusnak, and M. Sakaitani.** 1990. Molecular studies on enzymes in chorismate metabolism and the enterobactin biosynthetic pathway. Chem. Rev. **90:**1105–1129.
28. **Woods, D. D.** 1940. The relationship of *p*-aminobenzoic acid to the mechanism of the action of sulphanilamide. Br. J. Exp. Pathol. **21:**74–90.
29. **Ye, Z.-Z., J. Liu, and C. T. Walsh.** 1990. *p*-Aminobenzoate biosynthesis in *Escherichia coli*: purification and characterization of PabB as aminodeoxychorismate synthase and enzyme X as an aminodeoxychorismate lyase. Proc. Natl. Acad. Sci. USA **87:**9391–9395.
30. **Zalkin, H.** 1973. Anthranilate synthetase. Adv. Enzymol. **38:**1–39.
31. **Zalkin, H.** 1993. The amidotransferases, p. 203–309. *In* A. Meister (ed.), Advances in enzymology and related areas of molecular biology, vol. 66. John Wiley & Sons, Inc., New York.